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# Mechanism of RNA Polymerase Action: Characterization of the DNA-Dependent Synthesis of Polyadenylic Acid

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Purified preparations of DNA-dependent RNA polymerase from *Escherichia coli* catalyse a DNA-dependent synthesis of long-chain polyadenylic acid when ATP is the only ribonucleoside triphosphate added to the reaction. Polyadenylic acid synthesis differs from DNA-dependent synthesis of complementary RNA in three important respects. First, the chain-length of the polyadenylic acid product is greater by 5 to 10 times than that of the deoxythymidylic acid sequence in DNA which serves as template. Secondly, addition of one, two or all three of the other ribonucleoside triphosphates to the reaction at low concentrations (less than $2 \times 10^{-6}$ M) brings about a striking inhibition of polyadenylic acid synthesis. In the presence of all four of the ribonucleoside triphosphates, no polyadenylic acid is produced. Inhibition by any given ribonucleoside triphosphate is dependent on the presence in the DNA template of the nucleotide base complementary to that inhibiting triphosphate. Finally, only single-stranded DNA serves to direct polyadenylic acid synthesis.

It is proposed that DNA-dependent polyadenylic acid synthesis is due to a reiterative copying of short sequences of thymidylic acid residues in the DNA template rather than to a separate polyadenylic acid polymerase. The inhibition of this synthesis by the other ribonucleoside triphosphates may be due to fixation of the enzyme on the template at a deoxynucleotide base complementary to the base of the inhibiting triphosphate, thus preventing further copying of thymidylic acid sequences. The relevance of these findings to the general mechanism of polynucleotide synthesis by this enzyme is discussed.

## 1. Introduction

DNA-directed RNA polymerase catalyses the synthesis of RNA, utilizing the four ribonucleoside triphosphates as nucleotidyl precursors (reviewed by Grunberg-Manago, 1962; Stevens, 1963). In this reaction DNA serves as a template in directing the synthesis of an RNA of complementary nucleotide sequence (Furth, Hurwitz & Goldmann, 1961; Weiss & Nakamoto, 1961). This sequence complementarity between template and product is maintained despite radical changes in the nucleotide composition (Furth *et al.*, 1961; Hurwitz, Evans, Babinet & Skalka, 1963) or secondary structure (Hurwitz, Furth, Anders & Evans, 1962; Wood & Berg, 1963) of the template. The observation that purified preparations of RNA polymerase from *Escherichia coli* catalyse a DNA-dependent synthesis of polyadenylic acid when

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ATP is the only ribonucleoside triphosphate added to the reaction (Stevens, 1961; Chamberlin & Berg, 1962) presented an apparent exception to this complementarity of template and product.

Two unique features of this DNA-dependent polyadenylic acid synthesis were: (1) addition of one, two or all three of the other ribonucleoside triphosphates to the reaction brought about a striking inhibition of polyadenylic acid synthesis. In the presence of all four of the ribonucleoside triphosphates, no polyadenylic acid was formed; (2) the polyadenylic acid product exceeded 60 adenylic acid residues in length. Since it seemed unlikely that this reaction was due to a separate polyadenylic acid polymerase in the RNA polymerase preparations used,† we proposed (Chamberlin & Berg, 1962) that DNA-dependent polyadenylic acid synthesis results from a complementary copying by RNA polymerase of short deoxythymidylic acid sequences such as are known to occur in DNA (Burton & Petersen, 1960; Shapiro & Chargaff, 1960), accompanied by some process of chain elongation. This process of chain elongation was assumed to be a consequence of "slipping"; i.e., a continuous displacement of the product from the short deoxythymidylic acid template sequence, resulting in the generation of unpaired thymidylate residues to serve as sites for the addition of new adenylic acid units. We shall henceforth refer to this process as the "reiterative copying" of a given template sequence. Inhibition of DNA-dependent polyadenylic acid synthesis by the other nucleoside triphosphates was thought to result from interference with reiterative copying. The recent demonstration by Falaschi, Adler & Khorana (1963) that deoxythymidylic acid oligomers containing as few as five nucleotidyl residues could be used by *E. coli* RNA polymerase to direct the synthesis of a long-chain polyadenylic acid product (50 to 100 residues) provides strong support for the existence of such a reiterative copying process.

In the present paper we report experiments which concern the marked effect of changes in the secondary structure of DNA on its ability to direct polyadenylic acid synthesis, some observations on the nature of the inhibition of DNA-directed polyadenylic acid synthesis by nucleoside triphosphates, and discuss the bearing of these findings on the mechanism of polynucleotide synthesis by RNA polymerase.

## 2. Materials and Methods

Unlabeled ribonucleoside triphosphates and [$^{14}$C]ATP were purchased from Schwarz Bioresearch, Mt Vernon, New York. [$\alpha$-$^{32}$P]CTP, [$^{14}$C]GTP and [$^{14}$C]CTP were prepared as before (Chamberlin & Berg, 1962). Ribonucleoside mono- and diphosphates were products of the Sigma Biochemical Corporation, St. Louis, Missouri. Unlabeled and $^{32}$P-labeled deoxyribonucleoside triphosphates were purchased from the California Corporation for Biochemical Research, Los Angeles, California, and prepared according to Lehman, Bessman, Simms & Kornberg (1958).

*E. coli* exonuclease I was prepared according to Lehman (1960). *E. coli* RNA polymerase was prepared as previously described (Chamberlin & Berg, 1962). Enzyme fraction 4 was used throughout; after elution from the DEAE column, the enzyme was precipitated from solution by the addition of 1·2 vol. of saturated ammonium sulfate solution, pH 8, and was dissolved in a solution containing 0·1 M-ammonium sulfate,

† Previous studies (Chamberlin & Berg, 1962) excluded the possibility that the DNA-dependent polyadenylic acid synthesis was due to polynucleotide phosphorylase or to a polyadenylic acid polymerase of the RNA-dependent type (August *et al.*, 1962; Gottesman *et al.*, 1962). Furthermore, there was no separation of the activities for RNA and for polyadenylic acid synthesis during the 150-fold purification of the enzyme. Finally, gradient chromatography of fraction 4 RNA polymerase on DEAE-cellulose or on hydroxylapatite, which gives rise in each instance to a single peak of protein and of enzyme, failed to separate the two activities (Chamberlin, 1963).

0·01 M-tris buffer, pH 8, 0·001 M-$MgCl_2$ and 0·005 M-glutathione, and stored in a liquid-nitrogen refrigerator. Samples stored in this manner showed no loss in activity after storage for more than 14 months.

The assay for formation of complementary RNA with polymerase measures the rate of conversion of $^{32}P$ or $^{14}C$ from a ribonucleoside triphosphate into an acid-insoluble form (Chamberlin & Berg, 1962). Enzyme dilutions were made with a solution containing 0·01 M-tris buffer, pH 8, 0·01 M-$MgCl_2$, 0·01 M-$\beta$-mercaptoethanol, 0·00005 M-EDTA and 1 mg/ml. of crystalline bovine serum albumin. The reaction mixture (0·25 ml.) contained: 10 $\mu$moles of tris buffer, pH 7·9 to 8·1, 0·25 $\mu$mole of $MnCl_2$, 100 m$\mu$moles each of ATP, UTP, GTP and CTP, 250 m$\mu$moles of salmon sperm DNA, 3·0 $\mu$moles of $\beta$-mercaptoethanol and 10 to 80 units of enzyme. One of the ribonucleoside triphosphates was labeled with from 300 to 2000 cts/min/m$\mu$mole. After incubation at 37°C for 10 min, the reaction mixture was chilled in ice, and 1·2 mg of serum albumin (0·03 ml.) was added, followed by 3 ml. of ice-cold 3·5% PCA.† The precipitate was dispersed, centrifuged for 5 min at 15,000 **g**, and washed twice with 3 ml. portions of ice-cold PCA. The residue was suspended in 0·5 ml. of 2 N-ammonium hydroxide solution, transferred to an aluminum planchet and, after drying, counted in a windowless gas-flow counter. The assay for polyadenylic acid formation was carried out under identical conditions except that GTP, UTP and CTP were omitted from the reaction mixture.

The following DNA preparations were obtained essentially as described in the accompanying references: DNA from calf thymus (Kay, Simmons & Dounce, 1952), DNA from T2 phage (Josse, Kaiser & Kornberg, 1961), DNA from $\lambda$ phage (Kaiser & Hogness, 1960), calf thymus apurinic acid (Tamm, Hodes & Chargaff, 1952), dAT copolymer (Schachman, Adler, Radding, Lehman & Kornberg, 1960) and high molecular weight dI, dG and dC homopolymers (Inman & Baldwin, 1964). Polydeoxythymidylic acid oligomers of known chain-length were prepared and isolated by the method of Tener, Khorana, Markham & Pol (1958). They were generously provided by Dr. A. Nussbaum, of Stanford University, and by Dr. H. G. Khorana, of the University of Wisconsin. Heat denaturation of DNA was carried out by heating the preparation for 15 min at 100°C at an ionic strength equal to or less than 0·1, and cooling the solution rapidly in an ice-bath.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Nucleic acid concentrations are expressed in terms of total nucleotide.

## 3. Results

### (a) *Effect of DNA composition and secondary structure on polyadenylic acid synthesis*

A puzzling feature of DNA-dependent polyadenylic acid synthesis has been the observation that samples of DNA from a given source could differ significantly in their ability to direct polyadenylic acid formation, although these same preparations were nearly equivalent in their ability to direct RNA synthesis (Chamberlin & Berg, 1962; J. Hurwitz, personal communication). The source of these differences became known when it was found that single- but not double-stranded DNA could direct polyadenylic acid synthesis. As shown in Table 1, DNA preparations isolated with care to preserve the native helical structure of the polymer fail to support polyadenylic acid synthesis. Heat denaturation of these same samples, however, produces a DNA which is highly active in this regard.

† Abbreviations used: PCA = perchloric acid; dAT = deoxyadenylate-deoxythymidylate copolymer; dI polymer = polydeoxyinosinic acid; dC polymer = polydeoxycytidylic acid; dG polymer = polydeoxyguanylic acid; dT polymer = polydeoxythymidylic acid. A similar notation is used for the polyribonucleic acid analogues (rA polymer, rU polymer, rC polymer and rG polymer). $dT_9$ oligomer is a polydeoxythymidylic acid with a chain-length of nine. The nomenclature used is essentially that set forth by Inman & Baldwin (1962). dAMP, dADP and dATP designate the deoxyadenosine-5′-mono-, di- and triphosphates, respectively; a similar notation is used for deoxythymidine (dT), deoxyuridine (dU), deoxycytidine (dC) and deoxyguanosine (dG).

This observation suggested that the polyadenylic acid synthesis observed previously using native calf thymus DNA (Chamberlin & Berg, 1962) was due to the presence of denatured DNA in these preparations. This possibility was tested by treating the calf thymus DNA with exonuclease I from *E. coli* (Lehman, 1960), which selectively degrades denatured DNA. As shown in Table 2, this treatment

TABLE 1

*Activity of native and heat-denatured DNA in directing polyadenylic and RNA synthesis*

| DNA | Treatment | Incorporation of [$^{14}$C]AMP into: RNA | polyadenylic acid (m$\mu$moles) |
|---|---|---|---|
| $\lambda$ phage | None | 1·8 | < 0·1 |
| | 15 min at 100°C | 2·1 | 7·2 |
| T2 phage | None | 2·3 | 0·3 |
| | 15 min at 100°C | 2·0 | 3·3 |

The rates of RNA and polyadenylic acid synthesis were measured by incorporation of [$^{14}$C]AMP as described in Materials and Methods. Where appropriate, 25 m$\mu$moles of $\lambda$ DNA or 120 m$\mu$moles of T2 DNA were added. 7 $\mu$g of RNA polymerase were used in each experiment.

TABLE 2

*Effect of pre-incubation with* E. coli *exonuclease I on the ability of DNA to support polyadenylic acid and RNA synthesis*

| Template | Treatment | Incorporation of [$^{14}$C]AMP into: RNA | polyadenylic acid (m$\mu$moles) |
|---|---|---|---|
| Calf thymus DNA | No diesterase | 3·0 | 1·4 |
| | Diesterase | 2·6 | 0·1 |
| Calf thymus DNA, heated 15 min at 100°C | No diesterase | 1·9 | 6·5 |
| | Diesterase | 0·1 | 0·1 |

To a reaction mixture (0·13 ml.) containing 10 $\mu$moles of tris, pH 8, 1 $\mu$mole of $MgCl_2$ and 25 m$\mu$moles of calf thymus DNA were added 20 units of *E. coli* exonuclease I (DEAE-fractionated enzyme, Lehman, 1960) where noted, and the solution was incubated for 2 hr at 37°C. At this time the action of the exonuclease was stopped by addition of 0·25 $\mu$mole of $MnCl_2$ (cf. Lehman, 1960) and the following components were added (final volume 0·25 ml.): 100 m$\mu$moles of [$^{14}$C]ATP, 100 m$\mu$moles each of GTP, UTP and CTP (not added when measuring polyadenylic acid formation), 3 $\mu$moles of $\beta$-mercaptoethanol and 8 $\mu$g of RNA polymerase. After 10 min at 37°C the reaction was stopped in the usual manner and assayed for polyadenylic acid formation or RNA synthesis as described in Materials and Methods.

eliminates the ability of the calf thymus DNA preparation to serve as primer for polyadenylic acid synthesis, but has little effect on its ability to direct the synthesis of complementary RNA. As expected, a similar treatment of heat-denatured DNA with the exonuclease I destroys its ability to direct both RNA and polyadenylic acid synthesis. Thus, although either single- or double-stranded DNA is used as template for RNA synthesis, only single-stranded DNA can direct the formation of polyadenylic acid.

The ability of various DNA preparations of widely differing nucleotide composition to support polyadenylic acid synthesis is shown in Table 3. Although the dI and dC polymers are effective templates for RNA polymerase, directing the synthesis of rC and rG polymers respectively (Table 4),† neither polymer supports polyadenylic

TABLE 3

*Ability of various polydeoxynucleotides to direct polyadenylic acid synthesis*

| DNA added | [$^{14}$C]AMP incorporation (m$\mu$moles) |
|---|---|
| Heat-denatured calf thymus DNA | 3·5 |
| Calf thymus apurinic acid | 0·9 |
| Heat-denatured $\lambda$ DNA | 2·1 |
| $dT_{13}$ oligomer | 9·1 |
| $dT_9$ oligomer | 2·2 |
| dAT copolymer | $<0·03$ |
| dI polymer | $<0·03$ |
| dC polymer | $<0·03$ |

The standard RNA polymerase assay system was used except that no GTP, UTP or CTP was added; the only polynucleotide added was that shown. [$^{14}$C]ATP had a specific activity of 600 cts/min/m$\mu$mole. The amounts of the different preparations added were: 50 m$\mu$moles of denatured calf thymus DNA; 20 m$\mu$moles of $\lambda$ DNA; 94 m$\mu$moles of $dT_9$ oligomer; 12 m$\mu$moles of $dT_{13}$ oligomer; 60 m$\mu$moles of apurinic acid deoxynucleotide; 15 m$\mu$moles of dAT copolymer; 5 m$\mu$moles of dI polymer; and 5 m$\mu$moles of dC polymer. 4 $\mu$g of RNA polymerase were used in each experiment.

TABLE 4

*RNA synthesis directed by dI and dC polymers*

| Template | [$^{14}$C]nucleotide added | Incorporation (m$\mu$moles) |
|---|---|---|
| dC | GTP | 10·0 |
| | ATP | 0·18 |
| | CTP | 0·15 |
| | UTP | $<0·10$ |
| dI | GTP | $<0·10$ |
| | ATP | $<0·03$ |
| | CTP | 21·0 |
| | UTP | $<0·10$ |

The standard system and assay procedure were used except that, in the dC-directed reaction, 5 m$\mu$moles of dC and 73 $\mu$g of enzyme fraction 4 were incubated 45 min at 37°C. In the dI-directed reaction 4·5 m$\mu$moles of dI were incubated with 30 $\mu$g of enzyme for 20 min.

The specific activities of the $^{14}$C-labeled nucleotide substrates were: UTP and GTP, 200 cts/min/m$\mu$mole; CTP, 300 cts/min/m$\mu$mole; ATP, 500 cts/min/m$\mu$mole.

acid formation. Therefore, the DNA requirement in polyadenylic acid formation is not simply a requirement for a single-stranded polydeoxynucleotide. Parenthetically, it should be noted that the failure of these DNA homopolymers to direct synthesis of RNA homopolymers other than those predicted on the basis of Watson–Crick pairing

† The dG polymer failed to support a detectable incorporation of any of the four ribonucleoside triphosphates. This result may be due to the ability of polyguanylic acid to form complexes with an extremely stable, highly ordered secondary structure (Ralph *et al.*, 1962; Gellert *et al.*, 1962).

between template and product further supports the notion that under these conditions addition of a ribonucleotidyl unit to a growing RNA chain occurs only under the direction of a complementary nucleotide base in the DNA template. No polyadenylic acid formation was observed with dAT copolymer even though half of its nucleotide bases are thymine residues. This may be the result either of its alternating nucleotide sequence, or of its helical secondary structure. The ability of apurinic acid to direct polyadenylic acid synthesis is noteworthy, because this material contains only the thymine and cytosine residues of the original DNA attached to the phosphodiester backbone. These results support the view that polyadenylic acid synthesis is dependent on the presence of sequences of thymidylic acid residues in the DNA template.

### (b) *Inhibition of DNA-directed polyadenylic acid synthesis by the other ribonucleoside triphosphates*

#### (i) *High-efficiency inhibition*

One remarkable feature of polyadenylic acid formation by RNA polymerase is the marked decrease in the rate of synthesis observed on adding low concentrations of UTP, GTP or CTP to the DNA-directed reaction (Fig. 1). The inhibition observed with any single ribonucleoside triphosphate reached 30 to 40% as the concentration of that triphosphate was increased to about $1 \times 10^{-6}$ M and then remained nearly constant over a further fivefold increase in inhibitor concentration. Half-maximal inhibition was achieved at about $2 \times 10^{-7}$ M.

Because of the extremely low concentrations of triphosphate necessary to give the inhibition shown, this phenomenon has been designated *high-efficiency inhibition*. This inhibition is not affected by a fivefold increase in the concentration of the divalent metal ions in the reaction; similarly, no effect was seen when the DNA primer concentration was varied over a tenfold range (50 to 500 mμmoles per assay). The denatured DNA primer was present in saturating concentrations in all experiments. Thus, it does not seem that the inhibitory effect is mediated through the availability of the metal ion, or by lowering the affinity of the enzyme for the DNA primer. Furthermore, an increase in the ATP concentration from 1 to $3 \times 10^{-3}$ M did not change the inhibition of DNA-directed polyadenylic acid synthesis produced by $4 \times 10^{-6}$ M-GTP. This suggests that high-efficiency inhibition by any of the three nucleoside triphosphates is not due to simple competition with ATP for a site on the enzyme.†

The inhibitory effects of the individual ribonucleoside triphosphates were found to be independent and roughly additive (Table 5). Thus, with a denatured DNA primer, increasing the concentration of a given triphosphate from 2 to $4 \times 10^{-6}$ M did not significantly change the level of inhibition (Fig. 1), whereas addition of a second ribonucleoside triphosphate at $2 \times 10^{-6}$ M nearly doubled that level of inhibition (Table 5). In the presence of all three remaining ribonucleoside triphosphates, there was almost complete inhibition of AMP polymerization. Under these latter conditions, i.e. the presence of all four nucleoside triphosphates, synthesis of complementary RNA can occur; however, in this experiment the concentrations of UTP, GTP and CTP in the reaction are so low (about $\frac{1}{75}$ of the apparent $K_m$ previously determined

† Formal kinetic analysis of high-efficiency inhibition of DNA-directed polyadenylic acid formation was handicapped by the presence of low-efficiency inhibition (see following text), and also by substrate inhibition which was seen at concentrations of ATP in excess of 1 to $2 \times 10^{-3}$ M.

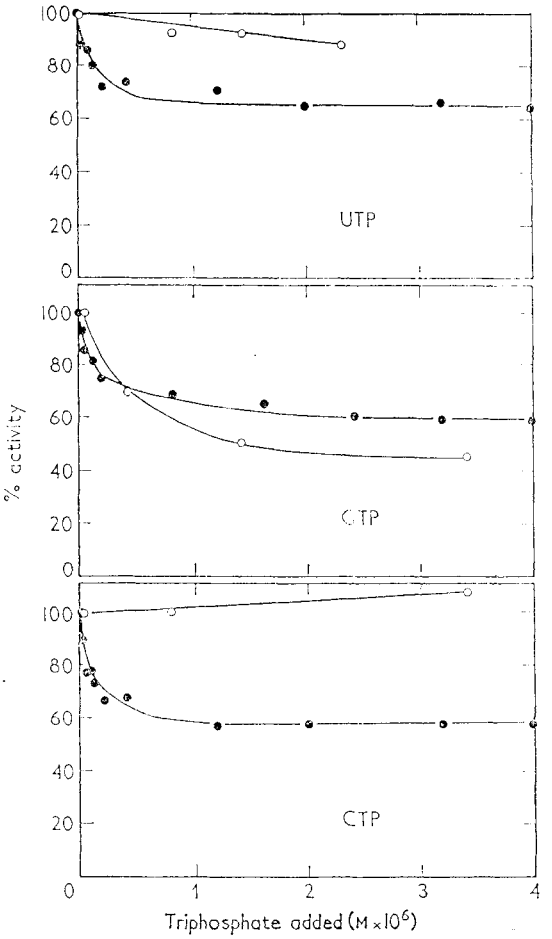


FIG. 1. *Inhibition of DNA-directed and apurinic acid-directed polyadenylic acid synthesis by UTP, GTP and CTP at low concentrations.* The standard RNA polymerase assay conditions were used, except that 15 mμmoles of denatured calf thymus DNA or 46 mμmoles of apurinic acid were added, and the only nucleotides added were [$^{14}$C]ATP ($4 \times 10^{-4}$ M) and either UTP, GTP or CTP at the concentrations shown. 3 μg of enzyme were added and 5·3 mμmoles of polyadenylic acid were formed in the absence of any inhibitor. —○—○—, apurinic acid primer; —●—●—, denatured DNA primer.

by Chamberlin & Berg, 1962) that the actual rate of synthesis of complementary RNA is negligible. Thus, in the presence of exceedingly small amounts of all three of the other ribonucleoside triphosphates, the DNA-dependent synthesis of polyadenylic acid is completely abolished.

(ii) *Effect of DNA composition on high-efficiency inhibition*

A clue to the mechanism of this high-efficiency inhibition of DNA-directed polyadenylic acid synthesis was provided by experiments in which DNA polymers of restricted base composition were used as templates (Table 6). With $dT_9$ oligomer as template, none of the other ribonucleoside triphosphates gave high-efficiency inhibition of polyadenylic acid formation; similarly, addition of non-complementary ribonucleoside triphosphates—that is, ribonucleoside triphosphates for which there

TABLE 5

*Additive inhibition by UTP, GTP and CTP of DNA-directed polyadenylic acid synthesis*

| Inhibitors added | [$^{14}$C]AMP incorporation |
|---|---|
| | % |
| None | 100 |
| GTP | 56 |
| UTP | 65 |
| CTP | 52 |
| GTP, UTP | 25 |
| GTP, CTP | 27 |
| UTP, CTP | 36 |
| GTP, UTP, CTP | 4 |

The standard RNA polymerase assay system was used except that 150 mμmoles of denatured calf thymus DNA were used as template, and the only ribonucleoside triphosphates added were [$^{14}$C]ATP and those indicated above. ATP was present at $4 \times 10^{-4}$ M concentration, and the other triphosphates were each present at $2 \times 10^{-6}$ M concentration. 3 μg of RNA polymerase were used and 5·4 mμmoles of polyadenylic acid were formed in the absence of inhibitor.

TABLE 6

*Effects of non-complementary ribonucleoside triphosphates on RNA homo- and copolymer synthesis*

| DNA template | Substrate | Inhibitor concentration (M) | % activity remaining with: GTP | UTP | CTP | ATP |
|---|---|---|---|---|---|---|
| dI polymer | [$\alpha$-$^{32}$P]CTP ($4 \times 10^{-4}$ M) | $4 \times 10^{-4}$ | 100 | 87 | — | 77 |
| | | $2 \times 10^{-3}$ | 20 | 23 | — | 15 |
| dC polymer | [$^{14}$C]GTP ($1 \times 10^{-4}$ M) | $1 \times 10^{-4}$ | — | 93 | 98 | 100 |
| dAT polymer | [$^{14}$C]ATP + UTP ($2 \times 10^{-4}$ M each) | $2 \times 10^{-4}$ | 96 | — | 100 | — |
| | | $8 \times 10^{-4}$ | 56 | — | 71 | — |
| | [$^{14}$C]ATP + UTP ($1{\cdot}2 \times 10^{-5}$ M each) | $8 \times 10^{-4}$ | 29 | — | 38 | — |
| $dT_9$ oligomer | [$^{14}$C]ATP ($4 \times 10^{-4}$ M) | $2 \times 10^{-4}$ | 100 | 82 | 110 | — |
| | | $2 \times 10^{-3}$ | 26 | 7 | 40 | — |
| | | $4 \times 10^{-3}$ | 1 | 2 | 3 | — |

The standard RNA polymerase assay system was used except for the DNA and ribonucleoside triphosphate components added, which were as listed in the Table. The amount of polydeoxynucleotide added, and the amount of polyribonucleotide produced in the absence of inhibiting triphosphates was: 8 mμmoles of dI polymer added, 0·5 mμmole of rC polymer produced; 20 mμmoles of dC polymer added, 10·7 mμmoles of rG polymer produced; 94 mμmoles of $dT_9$ oligomer added, 2·2 mμmoles of rA polymer produced; 22 mμmoles of dAT copolymer added, 56 and 9 mμmoles of rAU copolymer produced at [ATP] = $2 \times 10^{-4}$ M and $1{\cdot}2 \times 10^{-5}$ M, respectively. 6 μg of RNA polymerase were used in each experiment with the homopolymers and 1·5 μg of enzyme were used with dAT copolymer.

is no Watson–Crick complementary base in the template—gave no inhibition of the synthesis of rC, rG or rAU polymers directed by dI, dC or dAT polymers, respectively, when the concentrations of these triphosphates were equal to the concentration of the substrate triphosphates. (The significance of the inhibition observed at higher concentrations of non-complementary ribonucleoside triphosphates will be discussed in a later section.) Finally, and most important, with apurinic acid—which contains only thymine and cytosine as deoxynucleotide bases—high-efficiency inhibition of polyadenylic acid formation occurred only with GTP (Fig. 1). These results indicate that high-efficiency inhibition is intimately linked to the presence in the DNA template of the particular nucleotide base complementary to the inhibiting ribonucleoside triphosphate.

(iii) *High-efficiency inhibition by other nucleotides*

High-efficiency inhibition may not be restricted to the ribonucleoside triphosphates; ribonucleoside diphosphates and deoxynucleoside triphosphates also gave significant levels of inhibition at low concentrations, although the actual percentage of inhibition obtained was in general somewhat less than that obtained with the corresponding ribonucleoside triphosphate (Table 7). In some cases (e.g. dTTP and UDP) the

TABLE 7

*Inhibition of DNA-directed polyadenylic acid synthesis by low concentrations of deoxyribonucleoside triphosphates and ribonucleoside diphosphates*

| Inhibitor added | AMP incorporation % |
|---|---|
| None | 100 |
| GTP | 66 |
| GDP | 58 |
| dGTP | 75 |
| CTP | 57 |
| CDP | 62 |
| dCTP | 74 |
| UTP | 71 |
| UDP | 87 |
| dTTP | 93 |

The standard RNA polymerase assay conditions were used except that 150 mμmoles of denatured calf thymus DNA were used as primer and the only nucleotide added was [$^{14}$C]ATP ($4 \times 10^{-4}$ M) unless specified otherwise. Inhibiting nucleotides were all present at $1{\cdot}2 \times 10^{-6}$ M final concentration. 3 μg of enzyme were added and 5·6 mμmoles of polyadenylic acid were formed in the absence of any inhibitor.

analogues gave very little inhibition at the concentrations tested. It is not known whether the deoxyribonucleoside and diphosphate derivatives are active *per se*, but simply less inhibitory in certain cases, or whether the activity observed in these cases is due to contamination with ribonucleoside triphosphates. The latter explanation seems less likely, since the concentrations of, for example, dCTP required to produce a half-maximal inhibition were quite similar to those required for CTP.

Although we had previously reported that the deoxyribonucleoside triphosphates would not replace the ribonucleoside triphosphates with the *E. coli* RNA polymerase

it seemed desirable in view of the above to confirm this observation using a labeled deoxynucleotide as substrate. The results, Table 8, show that dATP is used at less than 2 to 3% of the rate of ATP for complementary RNA synthesis with either native or denatured DNA, and at less than 1% of the rate of ATP for polyadenylic acid formation. Although the deoxynucleotide preparations used had been treated with periodate (Lehman *et al.*, 1958) to remove ribonucleotide contaminants, it is still not possible to exclude the presence of a 2 to 3% contamination of the dATP with ATP, and hence the significance of this incorporation is questionable.

TABLE 8

*Utilization of dATP by RNA polymerase*

| Substrates | Concentration of triphosphates (M) | AMP incorporation with: Salmon sperm DNA (mμmoles) | Heated calf thymus DNA (mμmoles) |
|---|---|---|---|
| (1) [$^{14}$C]ATP, GTP, UTP, CTP | $4{\cdot}0 \times 10^{-4}$ | 4·9 | 2·2 |
| (2) [$^{32}$P]dATP, GTP, UTP, CTP | $3{\cdot}2 \times 10^{-4}$ | 0·08 | 0·04 |
| (3) Same as (2) | $6{\cdot}4 \times 10^{-4}$ | 0·12 | 0·06 |
| (4) Same as (3), no enzyme | $6{\cdot}4 \times 10^{-4}$ | — | <0·02 |
| (5) [$^{14}$C]ATP | $5{\cdot}0 \times 10^{-4}$ | — | 1·9 |
| (6) [$^{14}$C]dATP | $5{\cdot}0 \times 10^{-4}$ | — | <0·1 |

Standard assay conditions were used except as noted. In experiments 1 through 4, 250 mμmoles of salmon sperm DNA or calf thymus DNA were added with 6 μg of fraction 4 RNA polymerase. In experiments 5 and 6, 250 mμmoles of calf thymus DNA were added with 47 μg of enzyme and incubation was continued for 80 min at 37°C.

(iv) *Low-efficiency inhibition*

Addition of increasing amounts of UTP, GTP or CTP to the DNA-directed system for polyadenylic acid synthesis ultimately led to a complete inhibition of polyadenylic acid formation (Fig. 2). This type of inhibition (low-efficiency inhibition) was not

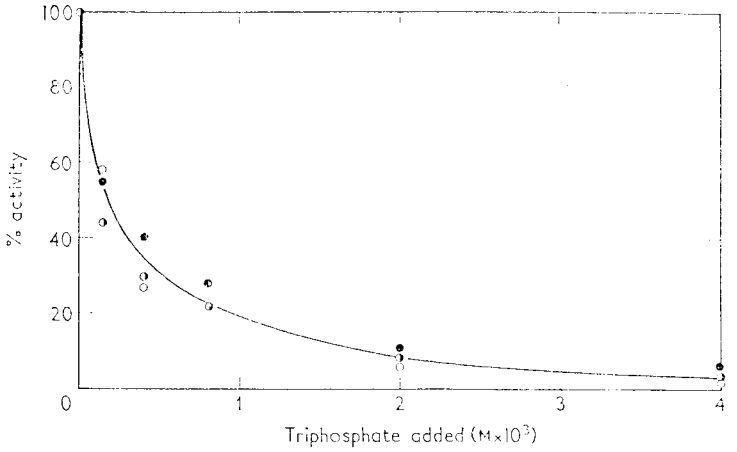


FIG. 2. *Inhibition of DNA-directed polyadenylic acid synthesis by UTP, GTP and CTP at high concentrations.* The standard RNA polymerase assay conditions were used, except that 150 mμmoles of heat-denatured calf thymus DNA were added, and the only nucleotides added were [$^{14}$C]ATP ($4 \times 10^{-4}$ M) and either UTP, GTP or CTP at the concentrations shown. 3 μg of enzyme were added and 5·3 mμmoles of polyadenylic acid were formed in the absence of any inhibitor. —◑—◑—, UTP added; —○—○—, GTP added; ---●--●—, CTP added.

restricted to polyadenylic acid formation, but occurred during polynucleotide synthesis with all templates of restricted base composition when non-complementary triphosphates were added at concentrations in excess of the substrate triphosphates (Table 6).

To avoid possible difficulties introduced by the presence of high-efficiency inhibition, it was convenient to study the inhibition of rAU copolymer synthesis by GTP and CTP as an example of low-efficiency inhibition. The inhibition was not relieved by a fourfold increase in the concentration of divalent metal ion or a fivefold increase in the concentration of template. As in the case of high-efficiency inhibition, the deoxyribonucleoside triphosphates and ribonucleoside diphosphates were also active as inhibitors (Table 9). The ribonucleoside monophosphates did not inhibit either

TABLE 9

*Inhibition of rAU synthesis by various non-complementary nucleotides*

| Experiment | Inhibitor added | rAU synthesis (mμmoles) | % |
|---|---|---|---|
| (1) | — | 55·6 | 100 |
| | CTP | 39·4 | 71 |
| | dCTP | 37·8 | 68 |
| | CDP | 38·0 | 69 |
| | GTP | 31·2 | 56 |
| | dGTP | 29·4 | 53 |
| | GDP | 40·0 | 72 |
| (2) | — | 9·0 | 100 |
| | CTP | 3·4 | 38 |
| | dCTP | 5·0 | 56 |
| | CDP | 4·8 | 54 |
| | GTP | 2·6 | 29 |
| | dGTP | 2·6 | 29 |
| | GDP | 5·6 | 62 |

The standard RNA polymerase assay was used except that the only nucleotide components added were those shown. Each incubation contained 22 mμmoles of dAT copolymer and 1·5 μg of RNA polymerase. In experiment 1, the concentration of [$^{14}$C]ATP and UTP was $2 \times 10^{-4}$ M each and in experiment 2 the concentrations were $1{\cdot}2 \times 10^{-5}$ M each. Inhibiting nucleotides were added at $8 \times 10^{-4}$ M in each case.

complementary RNA or polyadenylic acid synthesis at the highest concentrations tested ($2 \times 10^{-3}$ M). Low-efficiency inhibition by a triphosphate appeared to be competitive with respect to the substrate triphosphates. Kinetic analysis of the inhibition of rAU copolymer synthesis by GTP and CTP (Fig. 3) using the method of Lineweaver & Burk (1934) showed that in the presence of inhibitor the apparent $K_m$ for the substrate triphosphates was increased, but there was no change in the maximal rate of rAU synthesis. By this method, apparent $K_i$ values of $4{\cdot}4 \times 10^{-4}$ and $9{\cdot}7 \times 10^{-4}$ M were determined for GTP and CTP, respectively. $K_m$ values for ATP ranged from $8{\cdot}5 \times 10^{-5}$ to $1{\cdot}3 \times 10^{-4}$ M in repeated experiments. The $K_i$ found for the inhibition of rAU copolymer synthesis by GTP appeared to be nearly constant at different substrate concentrations (using the method of Dixon (1953) to determine $K_i$), whereas the value determined for CTP decreased from $9{\cdot}7 \times 10^{-4}$ to $3 \times 10^{-4}$ M as the concentrations of ATP and UTP in the reaction decreased from $2 \times 10^{-4}$ to $4 \times 10^{-6}$ M.

The method of Dixon (1953) allowed an analysis of the low-efficiency inhibition of DNA-dependent polyadenylic acid synthesis by UTP, GTP or CTP in the presence of high-efficiency inhibition. When the reciprocal rate observed at high concentrations of inhibitor with denatured DNA template was plotted against the concentration of inhibitor, a linear relationship resulted over the range of inhibitor concentrations

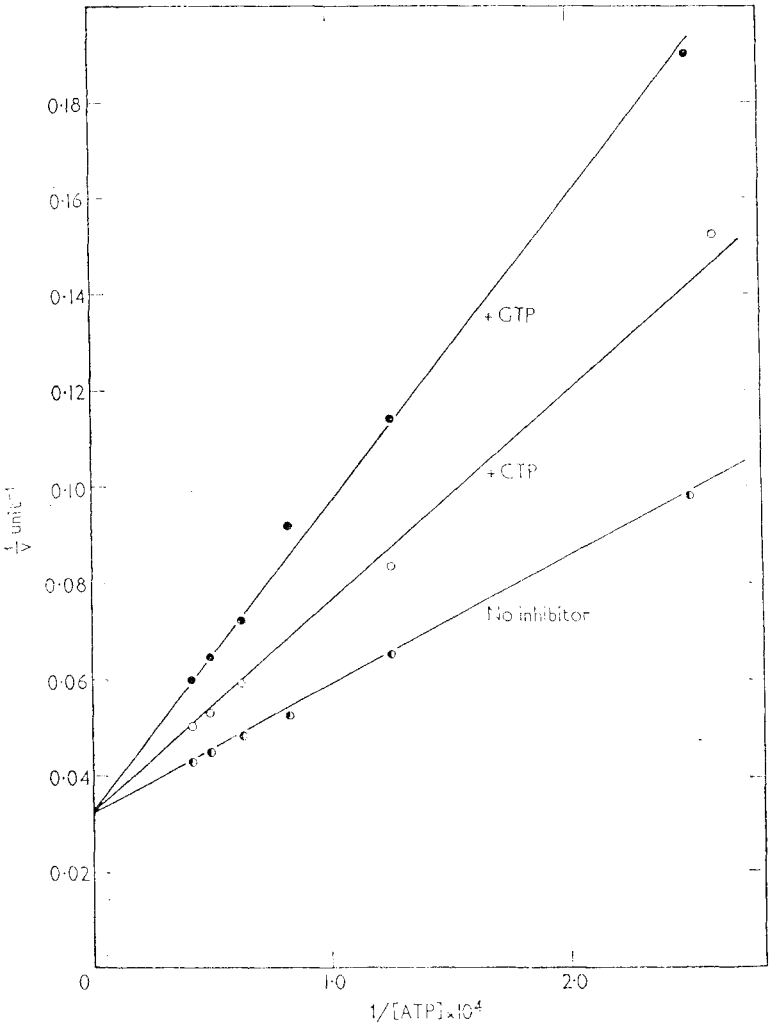


FIG. 3. *Kinetic analysis of the inhibition of rAU synthesis by GTP and CTP.* The figure shows e reciprocal rate of AMP incorporation (1 unit = 1 mμmole of [$^{14}$C]AMP incorporated per 1 hr) plotted against the reciprocal of ATP concentration. The standard RNA polymerase assay system was used except that dAT copolymer and the nucleotide components shown were added. Each incubation contained 22 μmoles of dAT copolymer and 1·5 μg of RNA polymerase. CTP and GTP were added, where indicated, at $6 \times 10^{-4}$ M, and the UTP concentration was kept equal to the ATP concentration. The $K_m$ calculated for ATP was $8{\cdot}5 \times 10^{-5}$ M.

from $8 \times 10^{-5}$ to $4 \times 10^{-3}$ M. By subtracting the fraction of the inhibition due to the high-efficiency inhibition from the total inhibition observed at each concentration, it was found that the average value of the low-efficiency $K_i$ for each triphosphate was from 2·5 to $4 \times 10^{-4}$ M. When $dT_9$ oligomer was used as primer, the relationship

obtained by plotting the reciprocal rate against inhibitor concentration was non-linear; the efficiency of inhibition increased markedly above an inhibitor concentration of $4 \times 10^{-4}$ M (see Table 5) until at $4 \times 10^{-3}$ M inhibitor concentration less than 5% of the original activity remained. The significance of this discrepancy and of the apparent difference between GTP and CTP in the inhibition of rAU copolymer synthesis is not presently known.

Although the detailed features of low-efficiency inhibition require further study, the competitive nature of this process suggests that the inhibition may be due to competition between the inhibiting triphosphate and the substrate triphosphate for a common site on the enzyme. Apparently simple binding of a nucleotide to the enzyme is not dependent on the presence of a complementary nucleotide base in the DNA template. Furthermore, the specificity of this site is apparently not directed toward the sugar residue of the nucleotide or the terminal phosphate of the triphosphate moiety.

## 4. Discussion

The results reported here and those of Falaschi *et al.* (1963) support the hypothesis that DNA-dependent formation of polyadenylic acid by RNA polymerase results from reiterative copying of sequences of thymidylate residues in a single-stranded DNA template. In this important respect, therefore, polyadenylic acid synthesis and complementary RNA synthesis are identical; in both, the specificity for polymerization of the nucleotide units resides in the base sequence of the template. However, three features of DNA-directed polyadenylic acid synthesis deserve comment: first, there is the disparity between the length of the product and the template; secondly, polyadenylic acid synthesis is inhibited by extremely low concentrations of ribonucleoside triphosphates; and finally, polyadenylic acid synthesis requires a single-stranded DNA as template. It is to these unique features that we will now direct our attention.

### (a) *Reiterative copying by RNA polymerase*

Whether the template is a short dT oligomer or a sequence of deoxythymidylic acid residues in single-stranded DNA, the resulting polyadenylic acid strand exceeds the length of the template sequence by as much as 10 to 20 times (Chamberlin & Berg, 1962; Falaschi *et al.*, 1963). While the existence of very long sequences of deoxythymidylic acid residues in DNA cannot be unambiguously excluded (Shapiro & Chargaff, 1960; Burton & Petersen, 1960), in DNA from phage $\phi$X 174, which is an effective primer for polyadenylic acid formation, the longest sequence of pyrimidine residues is 11 (Hall & Sinsheimer, 1963).

Hypothetically, synthesis of poly A might result from two sequential processes, the first a copying of the deoxythymidylic acid sequence, and the second, which could be independent of the primer, a chain-lengthening process. In practice, it has not been possible to bring about lengthening of pre-existing polyadenylic acid chains in the absence of DNA (Chamberlin & Berg, 1962) and hence there is no reason to suppose that the reactions, copying plus chain lengthening, are not one and the same. It is, therefore, attractive to suppose that chain lengthening is simply the manifestation of reiterative copying of a given template deoxythymidylic acid sequence. This could result from an interruption of the adenine to thymine base pairs produced during copying of the deoxythymidylic acid sequence, so that unpaired

thymine residues are exposed at the growing terminus of the polyadenylic acid chain (Fig. 4(A)). Thus, by the alternate processes of "slippage" and copying, reiterative copying of a given deoxythymidylic acid sequence would be achieved (Chamberlin & Berg, 1962).

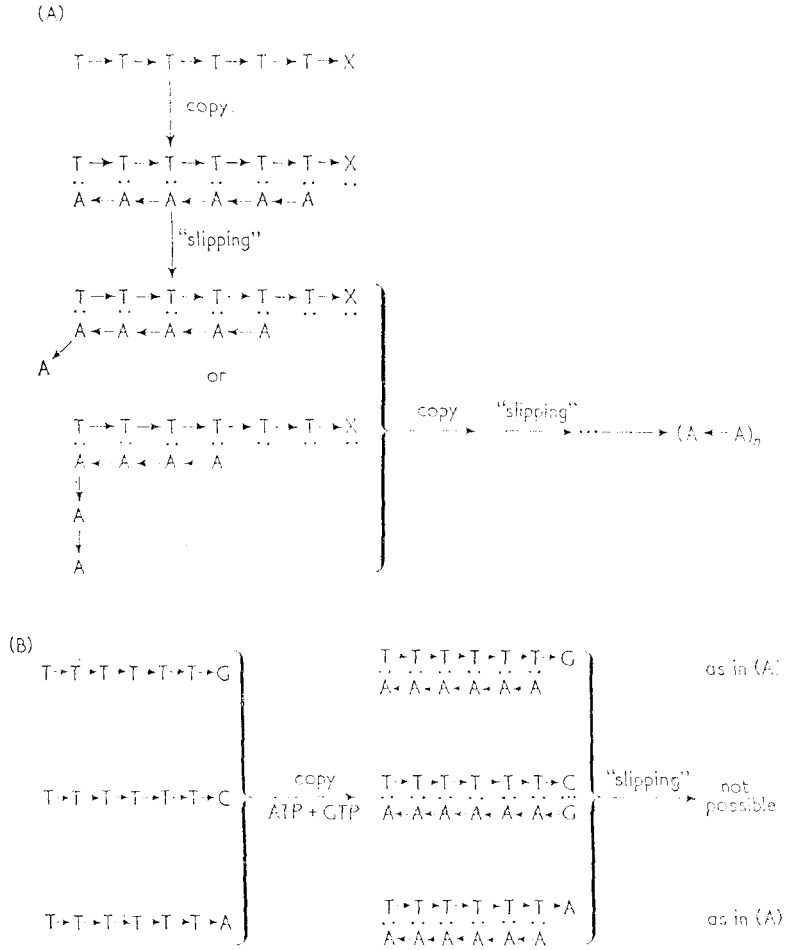


FIG. 4. *A schematic model for the mechanism of reiterative copying* (A) *and of high-efficiency inhibition* (B).

A somewhat analogous reiterative copying of an oligomeric template has also been observed to occur with the *E. coli* DNA polymerase (Kornberg, Bertsch, Jackson & Khorana, 1964). Oligomers consisting of alternating deoxyadenylic acid and deoxythymidylic acid residues, ranging in chain-length from eight to fourteen residues, were found to direct the synthesis of the long-chain copolymer dAT (Schachman, Adler, Radding, Lehman & Kornberg, 1960) without themselves being covalently incorporated into the growing dAT chain.

In the example noted above, as in the case of DNA-directed polyadenylic acid synthesis, the product can form a stable intramolecular structure, in this case the highly ordered dAT helix (Inman & Baldwin, 1962; Davies & Baldwin, 1963). It

may be that intramolecular complex formation by the nascent polynucleotide strand is an essential feature of reiterative copying. Thus, a weakening of template-to-product bonds, leading to a slippage process such as that postulated in Fig. 4(A), could be facilitated by the formation within the product molecule of an ordered structure. Such a phenomenon might explain why:

(1) polyuridylic acid synthesis, which probably results from copying of complementary deoxyadenylic acid sequences in the DNA template, occurs at only 5 to 10% of the rate of polyadenylic acid synthesis (Chamberlin & Berg, 1962). Although polyadenylic acid chains interact readily to form highly ordered, helical structures (Fresco & Klemperer, 1959), polyuridylic acid has no apparent secondary structure at temperatures above 10°C (see Lipsett, 1960);

(2) while $dT_{10}$ oligomer is a good template for polyadenylic acid synthesis, $dA_{10}$ oligomer fails to support polyuridylic acid synthesis (Falaschi *et al.*, 1963).

### (b) *Inhibition of DNA-dependent polyadenylic acid synthesis by GTP, UTP and CTP*

The second notable feature of the DNA-dependent synthesis of polyadenylic acid is the marked inhibition of this synthesis by the addition of low concentrations (less than $10^{-6}$ M) of the other ribonucleoside triphosphates. This is not a simple competition for a common site on the enzyme *per se* since, for example, dAT-directed rAU synthesis is unaffected by a 100-fold higher concentration of CTP than is required to give a maximal inhibition of DNA-directed polyadenylic acid synthesis. Moreover, this inhibition is not a necessary characteristic of reiterative synthesis, since RNA polymerase-catalysed polyadenylic acid synthesis directed by the dT oligomers as templates is not affected by any of the ribonucleoside triphosphates at concentrations less than $10^{-4}$ M (when the substrate triphosphate concentration is $4 \times 10^{-4}$ M).

High-efficiency inhibition is non-competitive with respect to substrate and template, and reaches a limiting value of 30 to 40% with any one triphosphate; with more than one triphosphate, the inhibitions are approximately additive. Since GTP, but not CTP or UTP, shows high-efficiency inhibition of polyadenylic acid synthesis with apurinic acid as template, we conclude that such inhibition occurs only when the deoxynucleotide base complementary to the inhibitor is present in the template. This fact, taken with the unusual characteristics of the inhibition, e.g. partial yet non-competitive, suggests that polyadenylic acid synthesis is completely blocked by any single triphosphate at some, but not all, of the deoxythymidylic acid sequences serving as template. Specifically, it seems likely that a given ribonucleoside triphosphate inhibits polyadenylic acid synthesis only at a sequence of deoxythymidylic acid residues which is terminated by a base complementary to the inhibitor. For example, copying of the sequence $(pT)_npC$ would only be inhibited by GTP; CTP and UTP would have no effect on polyadenylic acid synthesis at this sequence, but they would inhibit at $(pT)_npG$ and $(pT)_npA$ sequences, respectively.

One simple explanation of high-efficiency inhibition is that the inhibitory nucleotidyl moiety is incorporated at the end of the growing polyadenylic acid chain and that this prevents slippage because the inhibitor base cannot become hydrogen-bonded to the adjacent thymine residue (see Fig. 4(B)). This notion, however, does not account for the observation that deoxyribonucleoside triphosphates and

ribonucleoside diphosphates, which do not serve as substrates for RNA polymerase, produce high-efficiency inhibition. Moreover, since high-efficiency inhibition occurs in the presence of excess DNA and is not reversed by adding more DNA, it cannot be due simply to inactivation of template molecules.

Therefore we might propose that high-efficiency inhibition results from the inactivation of an enzyme–template complex. Perhaps, during the normal copying process, advancement of the enzyme from a DNA base, $n$, which had just been copied, to the next base in the sequence, $n+1$, requires a binding to the enzyme of the ribonucleotidyl moiety complementary to base $n+1$. The next ribonucleotidyl residue to be built into the chain would then "pull" the enzyme along the DNA template. In the absence of the base complementary to the template base $n+2$, the enzyme would remain complexed to the $n+1$ base pair. Thus in the presence of ATP alone, RNA polymerase would remain on the terminal residue of a sequence of deoxythymidylic acid residues and, by reiterative copying, generate polyadenylic acid. However, if the enzyme were "pulled" away from this sequence by the nucleoside triphosphate complementary to the nucleotide base terminating the deoxythymidylic acid sequence, reiterative synthesis of polyadenylic acid synthesis would be blocked and the enzyme would remain bound at that site.

This model requires that during high-efficiency inhibition the enzyme–template complex be non-dissociable. One possibility is that the complex formed between RNA polymerase and the template DNA is non-dissociable even under the usual conditions of synthesis. Alternatively, it may be that only the ternary complex enzyme–inhibitor–template is non-dissociable. Resolution of this question will come from further study of the nature of the enzyme–template complex.

### (c) *The requirement for single-stranded DNA*

Whereas RNA polymerase utilizes either single- or double-stranded DNA as template for the synthesis of complementary RNA (Chamberlin & Berg, 1962), only single-stranded DNA directs polyadenylic acid formation. Since, statistically, sequences of deoxythymidylic acid residues should seldom, if ever, occur at the ends of the molecule, it appears that RNA polymerase is able to initiate copying at an interior point of a single-stranded DNA chain. This is also shown by the ability of single-stranded DNA from phage $\phi X$ 174 to act as template for RNA polymerase (Chamberlin & Berg, 1963,1964; Sinsheimer & Lawrence, 1964), since this DNA appears to have no free end.

Why is helical DNA inactive in directing polyadenylic acid formation? Since RNA polymerase has a much greater affinity for single- than for double-stranded nucleic acid (Hurwitz *et al.*, 1962; Chamberlin, 1963; Wood & Berg, unpublished observations), it may be that the enzyme is restricted in its attachment to single-stranded regions of the DNA molecule. Such regions exist at breaks in, or at the end of, the DNA helix (Rich & Tinoco, 1960) as a result of the end effect (Schellman, 1955). Thus RNA polymerase *in vitro* may initiate copying only at these loci. The probable restriction of deoxythymidylic acid sequences to the interior of the helix would therefore prevent a helical DNA molecule from being used as a template for polyadenylic acid synthesis. An alternative possibility is that this requirement for single-stranded DNA could be a unique feature of reiterative copying; in a helical structure, the deoxyadenylic acid sequence complementary to

the deoxythymidylic acid sequence being copied might rapidly displace the polyadenylic acid from the template sequence, thus preventing further reiterative copying of the sequence. A displacement of this sort has been postulated to occur during the synthesis of complementary RNA on helical templates (Chamberlin & Berg, 1963, 1964). A choice between these two alternatives must await further study. In particular, it is of great interest to know whether RNA polymerase *in vitro* has a restricted number of starting points on the DNA helix.

### (d) *The specificity of RNA synthesis*

In previous discussions we sought to explain the unique high-efficiency inhibition of RNA polymerase in terms of the normal mechanism of complementary RNA synthesis. It seems appropriate at this point to explore the consequences of such a model, particularly as they relate to our understanding of how the specificity and fidelity of the copying process is assured. While it is reasonable to assume that the specificity of the complementary copying process resides in the known hydrogen-bonding relationships between the complementary bases (see Crick, 1957), in a free equilibrium the strength of the interactions between a nucleotide base in DNA and a mononucleotidyl residue is not great enough to produce any significant concentration of base pairs (see Lipsett, Heppel & Bradley, 1961). Therefore, the enzyme must participate in forming and stabilizing the pairing. How does the enzyme carry out this process? Quite possibly the enzyme might first bind the ribonucleoside triphosphates in a reversible equilibrium at a common site. Evidence for such an initial, low-affinity binding is that any ribonucleoside triphosphate inhibits the synthesis of polymer in a manner which is competitive with the substrate triphosphate (low-efficiency inhibition).

Where the bound triphosphate is complementary to the next DNA base to be copied, that triphosphate is bound either irreversibly, or with a very high affinity, even if it cannot participate in phosphodiester bond formation. Thus, the concentration of a triphosphate required to give half-maximal inhibition of DNA-dependent polyadenylic acid synthesis is about $2 \times 10^{-7}$ M, suggesting that the affinity of the enzyme for a complementary triphosphate may be as much as 1000 to 2000 times greater than its affinity for a non-complementary triphosphate.† It seems unlikely that this large increase in affinity could result entirely from the formation of two hydrogen bonds, with a $\Delta F$ of about 3 kcal (Schellman, 1955), and it may be that a considerable part of this increase is actually due to a change in the structure of the enzymic site. Thus, although the specificity of the copying process resides in the ability of the incoming nucleotidyl unit to form a hydrogen-bonded base pair with the DNA template, the increase in affinity of the enzyme for the nucleotide which forms the correct pair could magnify this selectivity many-fold.

### (e) *What is the role of the DNA-dependent synthesis of polyadenylic acid?*

In addition to the DNA-dependent RNA polymerase, several other enzymes have been described which carry out the synthesis of polyadenylic acid. Thus, in the presence of ADP, polynucleotide phosphorylase produces polyadenylic acid. August *et al.* (1962) have isolated an RNA-dependent enzyme from *E. coli* ribosomes which

† If the interaction between inhibitor and enzyme in high-efficiency inhibition is irreversible, as expected from the models proposed above, then it is not valid to use the apparent $K_I$ values found for this inhibition as measures of the affinity of the enzyme for the triphosphates.

converts ATP to polyadenylic acid. A similar enzyme, apparently from the non-ribosomal fraction of *E. coli*, has been reported by Gottesman *et al.* (1962). Similar enzymes have also been isolated from calf thymus nuclei (Edmonds & Abrams, 1960, 1962), and from chick embryo chorioallantoic membrane (Venkataraman, 1962). In the former case, the enzyme shows a requirement for the presence of a polyribonucleotide isolated from the same source, which closely resembles polyadenylic acid (Edmonds & Abrams, 1962). Despite the widespread occurrence of these enzymes, and the suggestion that this polymer may exist *in vivo*, no function is known for polyadenylic acid, although it has been speculated that it may play some part in the control of protein synthesis (August *et al.*, 1962; Edmonds & Abrams, 1962). It seems doubtful that the DNA-dependent RNA polymerase accounts for a significant amount of polyadenylic acid synthesis *in vivo*, in view of the marked inhibition of this activity by extremely low concentrations of the other ribonucleoside triphosphates.

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